

PATENT SPECIFICATION

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- (21) Application No. 1922/73 (22) Filed 15 Jan. 1973
- (31) Convention Application No. 48 280 (32) Filed 11 Feb. 1972 in
- (33) Italy (IT)
- (44) Complete Specification published 18 April 1974
- (51) International Classification A61K 27/00//C07G 7/026
- (52) Index at acceptance
A5B 20X 20Y 280 281 28Y 31X 31Y 382 38Y 39X 778
C3H 2



(54) TREATMENT OF HEART DISEASES

(71) We, ISTITUTO FARMACOLOGICO SERONO, S.p.A an Italian Body Corporate, of Via Casilina, 125, Rome, Italy, do hereby declare the invention for which we
5 pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:

This invention relates to medicaments 10 and, more particularly, to the therapeutical use in a pharmaceutical preparation of a phosphoprotein existing in nature which is called "Phosvitin". In 1885 Bunge (Z. Physiol. Chem. 9,49:1885) isolated from 15 egg yolk an iron-containing phosphoprotein complex. A few years after, Hugueneng and Morel (Compt. Rend. 140, 1065; 1905) expressed the hypothesis that such a complex—which was named by them "haemotogen"—would be involved in the formation 20 of the fowl embryo haemoglobin. This substance awakened the interest of many scientists; only, however, in 1949 were Mecham and Olcott (J. Am. Chem. Soc. 71, 25 3670; 1949) able to isolate from egg yolk a homogenous phosphoprotein fraction that was named by them "Phosvitin". The identity of the former "haemotogen" with such an "iron-phosvitin" complex was demonstrated in 1964 by O. Greengard et al. (Biochim. Biophys. Acta 90, 406, 1964).

The presence of phosvitin having been demonstrated in hen's eggs, an obvious consequence was that it was sought for in 35 the eggs of other animals. Wallace et al. (Canad. J. Biochem. 44, 1647; 1966) isolated it from the eggs of vertebrates (tortoise, frog and so on); G. Schmidt et al. (Biochem. Biophys. Research Communications 18, 60, 40 1965) from the eggs of salmon; and Y. Macco et al. (J. Biol. Chem. 241, 3822; 1966) from the eggs of certain species of fish.

Further and deeper investigation was 45 devoted to searching for phosvitin in the
[Price 25p]

blood of hens and other animals since it was logical to suppose that its synthesis would occur in some organ and that phosvitin would be transferred from this organ to the egg yolk through the flow of blood. Thus, Mock et al. (Canad. J. Biochem. Physiol. 39,109; 1961) extracted phosvitin from the blood of non-laying hens to which estrogens had been administered, whereas Heald et al. (Biochem. J. 87, 571; 1963) demonstrated that a substance identical to the phosvitin existing in the egg yolk can be found in the blood of laying hens, even if the latter have not been treated with estrogens. Further studies showed that phosvitin is synthesized in the liver and that its formation is hormone-controlled, being stimulated by the presence of estrogens.

From the chemical point of view, phosvitin is a phosphoprotein, that is, a protein containing removable phosphate groups which can be easily removed by treatment with alkali and enzymes (phosphokinases).

In its molecule 17 amino acids have been identified, 50% of this amino acid content being serine; hexoses (about 2.5%) and glucosamine (1.4%) are also present. The estimated molecular weight of phosvitin is 40,000 to 50,000 (Allerton et al., J. Biol. Chem. 240,3892; 1965).

All percentages quoted in this specification are percentages by weight.

In the lyophilized state phosvitin appears as an off-white, odourless powder which is 80 soluble in water, 0.9% saline and 10% MgSO₄ solution, whereas it is insoluble in a solution containing less than 2.5% MgSO₄ and in the usual organic solvents. Its isoelectric point is at pH 1.8.

Phosvitin contains 9.6 ± 0.3% phosphorus, 11.6 ± 0.4% nitrogen and 0.3 to 0.4% iron. Molar ratio P/N = 2.5 to 2.9; ashes = 31 ± 2%.

A solution containing 0.16% phosvitin

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in 0.1 M potassium chloride at pH 6.5 to 6.7 has no significant absorption peak between $\lambda = 250$ and $\lambda = 290$ millimicrons. Paper electrophoresis (Whatman

5 registered Trade Mark—3 MM strips; 3 x 27 cm; 200 v-10 mA) of 0.3% phosvitin in borate buffer only shows a spot using the reagent specific to phosphate esters (blue spot), whereas Schwarz starch (a 10 reagent specific to proteins) does not show any further spots. A further characteristic feature is that when treated with 1% toluidine blue in 7% acetic acid the paper strip shows a blue coloured spot which 15 does not disappear when dipped in 7% acetic acid in methanol solution.

By following the usual procedure of polyacrylamide gel electrophoresis phosvitin gives one or more blue bands (which 20 are due to the formation of molecular aggregations) when treated with 1% toluidine blue in 7% acetic acid.

By column chromatography on DEAE-cellulose phosvitin is eluted as a single 25 homogenous fraction.

From the above information it can be clearly seen that phosvitin is a known substance which has been extensively studied by a number of investigators. No pharmacological-therapeutic actions of this substance have, however, been disclosed to date.

Surprisingly, investigations we have carried out have shown that phosvitin may 35 be used as a medicament particularly suitable for use in curing and/or preventing such diseases as myocardial (in particular dysmetabolic myocardial) diseases and coronary heart diseases, as well as for use 40 as an adjuvant in cases of heart failure.

Accordingly the present invention provides a pharmaceutical preparation comprising phosvitin together with a pharmaceutically acceptable carrier or diluent. 45 Preferably, the weight ratio phosvitin: carrier or diluent is from 1:1 to 1:0.1. In one embodiment the preparation is in lyophilized form and phosvitin is utilized with glycocoll, the weight ratio phosvitin: 50 glycocoll preferably being about 1:0.5.

An especially preferred embodiment of this invention is a pharmaceutical preparation suitable for daily injection in human beings which comprises from 0.1 to 1 g. 55 phosvitin as a daily dose.

The present invention also provides a process for preparing a pharmaceutical preparation for use in the treatment of heart diseases, which process comprises 60 dissolving phosvitin and a pharmaceutically acceptable carrier or diluent in a pyrogenic distilled water, filtering the solution through a sterilizing filter and lyophilizing the filtrate to obtain a pharmaceutical preparation 65 which is suitable for injection.

Preferably, in such a process the pharmaceutically acceptable carrier or diluent is glycocoll.

The acute toxicity of the medicament of this invention was determined in mice 70 intravenously, intraperitoneally and orally. The tests carried out on adult male Swiss mice having the average weight of 18 g. gave the following results:

$LD_{50} = 334.41$ mg/kg i.v. 75
 $LD_{50} = 4050$ mg/kg i.p.
 $LD_{50} > 8000$ mg/kg orally

The following specific Examples are now given to further illustrate the invention.

Examples 1 and 2 merely illustrate the 80 pharmacological properties of phosvitin and do not describe preparations in accordance with the invention.

EXAMPLE 1

Male albino Wistar rats having the 85 average weight of 240 g. were first anaesthetized with 35 mg/kg i.p. sodium pentobarbital (Nembutal) and then treated with 2 LU./kg vasopressin (Pitressin) by quick intravenous infusion, then given 90 phosvitin in doses of 1000 mg/kg i.p. or 750 mg/kg i.p. plus 250 mg/kg i.v.

In both cases a marked reduction or prevention of the main electrocardiographic changes induced by the intravenous 95 administration of vasopressin was observed.

EXAMPLE 2

Male albino Wistar rats having the 100 average weight of 240 g. were first brought into a state of hypoxia by forced ventilation of an air/nitrogen mixture (30:70 by volume) and treated with 2-3 mcg./kg i.v. nor-adrenaline, then given phosvitin in a dose of 1000 mg/kg i.p.

A marked reduction or prevention of the 105 main electrocardiographic changes induced by the intravenous administration of nor-adrenaline to animals brought into a state of hypoxia was observed.

EXAMPLE 3

An isolated guinea-pig atrium dipped in Ringer-Krebs-Henselait solution was made 110 hypodynamic by submitting it to prolonged electrical stimulation at the constant frequency of 2 stimuli per second (5 milliseconds per stimulus).

The increase in contraction amplitude induced by the addition of 1 mcg. per ml. of bath contents of either nor-adrenaline or adrenaline was markedly enhanced in 115 both cases by the addition of phosvitin in a dose of 25 mcg/ml of bath contents.

Further experiments showed that the medicament of the invention lacks any 120 coronarodilator activity.

From the above it clearly appears that phosvitin is a highly useful therapeutic tool in myocardial (in particular dysmetabolic myocardial) diseases, in coronary heart diseases, and as an adjuvant in cases 130

of heart failure.

A typical preparation of phosvitin prepared by extraction from hen's egg yolk is made as follows:

- 5 Several eggs were broken, the yolk being separated from the albumen and extracted several times with ethanol. The alcoholic phospholipid-containing liquor was put aside whereas the protein residue was
- 10 squeezed and extracted several times with acetone. The acetonate neutral fat (egg oil) containing liquor was put aside whereas the protein residue, deprived both of phospholipids and lipids, was extracted with
- 15 10% aqueous magnesium sulfate solution. Raw phosvitin was precipitated by diluting the saline solution with 3.4 volumes of water and the precipitate dissolved in 10% sodium chloride aqueous solution. Further
- 20 magnesium sulfate was added up to 10% concentration, the saline was then filtered and phosvitin was precipitated again by diluting the filtrate with 3.4 volumes of water. The thus-obtained phosvitin precipitate was first dissolved in 10% NaCl, the saline desalinated by means of dialysis, filtered and lyophilized.

Lyophilized phosvitin may be mixed with suitable carriers or diluents such as 30 mannos, lactose and glycocoll in a weight ratio phosvitin: carrier or diluent generally ranging from 1:1 to 1:0.1. In accordance with the invention it has been found that, among suitable carriers or diluents, glycocoll particularly helps solution of the lyophilized product in water or saline. The preferred phosvitin: glycocoll ratio is about 1:0.5. A preferred aspect of the present invention is a pharmaceutical preparation 40 in lyophilized form containing phosvitin and glycocoll in a weight ratio of about 1:0.5. The present pharmaceutical preparations may also contain a common antiseptic agent, e.g. sodium thimerosal (Merthiolate).

The following is a typical example of a method which may be used for preparing ampoules or vials containing a pharmaceutical preparation in accordance with the 50 invention and in lyophilized form.

EXAMPLE 4

An aqueous solution was prepared having the following composition:

Phosvitin	10 g.
Glycocoll	5 g.
Sodium Merthiolate	10 mg.

Apyrogenic distilled water up to 100 ml. The first three ingredients were dissolved in the water at room temperature (22 to 60 25°C) with stirring. The resulting solution was filtered through a sterilizing SEITZ EK filter and put into 3 ml. dark-glass ampoules or vials in an amount of 1 ml. per ampoule or vial. Freezing and 65 lyophilization were carried out subse-

quently. Thus, each ampoule or vial of lyophilized product contained 100 mg. phosvitin and 50 mg. glycocoll.

A pharmaceutically effective daily dose in man has been found to be in the range 70 of from 0.1 to 1 g. phosvitin. A preferred way of administration is by intramuscular or intravenous injection.

The invention includes a method for the treatment of heart disorders in a non-human animal such as myocardial diseases, coronary heart diseases, and heart failure, which method comprises administering to the non-human animal a pharmaceutically effective amount of phosvitin.

It should be clearly understood that we make no claim herein to solutions or suspensions of phosvitin in common solvents.

Subject to the foregoing disclaimer,

WHAT WE CLAIM IS:

1. A pharmaceutical preparation comprising phosvitin together with a pharmaceutically acceptable carrier or diluent.

2. A pharmaceutical preparation as 90 claimed in claim 1, wherein the weight ratio phosvitin: carrier or diluent is from 1:1 to 1:0.1.

3. A pharmaceutical preparation as 95 claimed in claim 1 or claim 2, which preparation is in lyophilized form and comprises phosvitin and glycocoll.

4. A pharmaceutical preparation as 100 claimed in claim 3, in which the weight ratio phosvitin: glycocoll is about 1:0.5.

5. A pharmaceutical preparation as 105 claimed in any one of the preceding claims additionally containing an antiseptic agent.

6. A pharmaceutical preparation as 110 claimed in claim 5, wherein the antiseptic agent is sodium thimerosal.

7. A pharmaceutical preparation as 115 claimed in any one of the preceding claims, which preparation is suitable for daily injection in human beings and comprises from 0.1 to 1 g. phosvitin as a daily dose.

8. A process for preparing a pharmaceutical preparation for use in the treatment of heart diseases, which process comprises dissolving phosvitin and a pharmaceutically acceptable carrier or diluent in apyrogenic distilled water, filtering the solution through a sterilizing filter and lyophilizing the filtrate to obtain a pharmaceutical preparation which is suitable for injection.

9. A process as claimed in claim 8, wherein the pharmaceutically acceptable carrier or diluent is glycocoll.

10. A process as claimed in claim 9, 125 substantially as hereinbefore described in Example 4.

11. A pharmaceutical preparation whenever prepared in a process as claimed in any one of claims 8 to 10.

12. A process as claimed in claim 8,
wherein the phosvitin component of the
preparation is prepared by a process sub-
stantially as hereinbefore described in
5 Example 3.

13. A pharmaceutical preparation as
claimed in claim 1, whenever prepared in
a process as claimed in claim 12.

14. A pharmaceutical preparation as
10 claimed in claim 1 substantially as herein-
before described in Example 3 or Example
4.

15. A method for the treatment of heart
disorders in a non-human animal such as
myocardial diseases, coronary heart diseases, 15
and heart failure, which method comprises
administering to the non-human animal a
pharmaceutically effective amount of
phosvitin.

16. A method as claimed in claim 5, 20
wherein the phosvitin is administered in
the form of a pharmaceutical preparation
as claimed in any one of claims 1 to 7,
11, 13 or 14.

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Printed for Her Majesty's Stationery Office by The Tweeddale Press Ltd., Berwick-upon-Tweed, 1974.
Published at the Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies
may be obtained.